31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Detection of circulating melanoma cells in human blood using photoacoustic flowmetry

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Abstract— **Detection of circulating tumor cells (CTC's) in human blood and lymph systems has the potential to aid clinical decision making in the treatment of cancer. The presence of CTC's may signify the onset of metastasis, indicate relapse, or may be used to monitor disease progression. A photoacoustic flowmetry system was designed and tested for detecting circulating melanoma cells (CMC's) by exploiting the broadband absorption spectrum of melanin within CMC's. The device was tested on cultured melanoma cells in saline suspension and in a Stage IV melanoma patient. The device showed a detection threshold of a single melanotic melanoma cell from culture. Transient photoacoustic events were detected in a sample derived from a Stage IV melanoma patient that corresponded to particles passing through the laser beam path, indicating the presence of single melanoma cells in the human circulatory system.**

I. INTRODUCTION

Detection of CTC's in human blood has the potential to aid clinical decision making in the treatment of cancer[1-3]. The presence of CTC's has been positively correlated to disease state and may be used to monitor disease progression. Other means are being tested for CTC detection, including Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), immunomagnetic separation, microfluidic separation, and laser flowmetry though their clinical implementation is not feasible due to complexity and sensitivity problems. Technical barriers for these procedures include non-repeatability, numerous opportunities for human error, as well as lengthy preparations and analysis, thus precluding their implementation as viable clinical tools. Indeed, CTC detection is still an uncertain research area and optimal detection has yet to be determined. The potential for utilizing the knowledge of CTC's in the circulatory or lymph system is great and new modalities must be explored to determine the best means for CTC detection.

The use of photoacoustics, or laser induced ultrasound, to detect CTC's in human blood samples presents a novel approach to cancer diagnostics. Photoacoustics occurs when the optical energy of a photon is transduced into a mechanical disturbance, resulting in an acoustic wave[4-8]. The photoacoustic effect is obtained from *transient thermoelastic expansion*, in which laser energy is deposited into a confined region of matter such that rapid heating occurs followed by rapid expansion. This expansion causes a mechanical fluctuation in the medium manifested as an acoustic wave detectable by precision pressure sensors.

In order to create photoacoustic waves using thermoelastic expansion, the targeted CTC must have some intrinsic optical absorber. While most cells are colorless in the visible wavelengths of light, melanocytes produce melanin, a broadband optical absorber. Melanoma cells are derived from melanocytes with as many as 95% of such cells containing melanin. Preliminary work in the photoacoustic detection of melanotic CTC's has proven successful, identifying small concentrations of cultured human melanoma cells *in vitro* using the endogenous absorption enabled by the melanosomes[8].

A depiction of the detection apparatus is given in Figure 1. The schematic represents melanoma cells interacting with laser energy thus propagating a pressure wave. The *photoacoustic waveforms* generated by the presence of melanoma within suspension are detected using a piezoelectric copolymer film used to transduce mechanical acoustic energy to a quantifiable voltage from which signal presence can be readily extracted.

II. MATERIALS AND METHODS

Experimental Apparatus

A frequency-tripled Nd:YAG laser pumping an optical parametric oscillator (Vibrant 355 II, Opotek, Carlsbad, CA) was employed to provide 450 nm laser light with a pulse duration of 5 ns. Beam energy entering the flow cell was 17–19 mJ with a pulse repetition rate of 10 Hz. Radiant exposure was 0.461J/ cm ^{2} with a spot size of 0.8x1.0 mm. Laser light was taken directly from polarizer and spatially oriented by a cylindrical lens (LJ1014L2-B, Thorlabs, Newton, NJ). The light beam was collimated using two plano-convex lenses, 100mm and 50mm focal length (LA1509 and LA1131, Thorlabs, Newton, NJ) before entering the flow cell. A peristaltic pump (Masterflex L/S Economy Drive, Cole-Parmer Instr., Chicago, IIL) and platinum-cured silicon tubing (L/S 14, Cole-Parmer Instr., Chicago, IL) facilitated the circulation of experimental solutions. A customized quartz flow cell (Spectrocell Inc., Oreland, PA) was used as an excitation and acoustic wave collection device. Cell suspensions were circulated at a rate of 0.15 ml/ s for approximately 30 s. Acoustic pressure waves were detected by a 100 μm piezoelectric polyvinylidene diflouride (PVDF) copolymer film (Ktech Corp., Albuquerque, NM) sealed on the lateral surface of a 5 mm diameter detection aperture of the flow cell. The targeted cells and resulting photoacoustic signals were

This work was supported by the Wallace H. Coulter Foundation.

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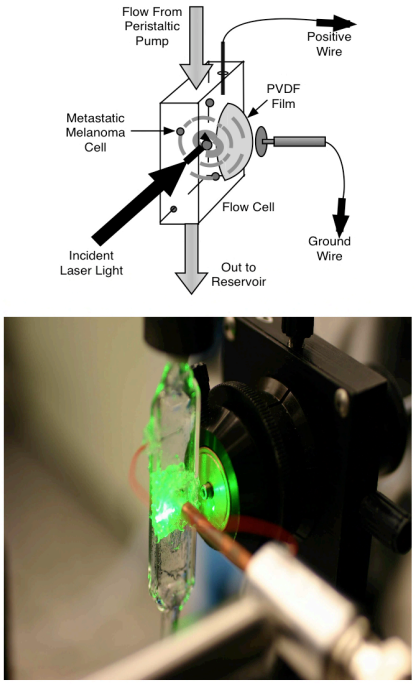

Fig. 1. **Photoacoustic apparatus.** (Top) Schematic of photoacoustic apparatus detecting human melanoma cells[8]. (Bottom) Photograph of photoacoustic device.

detected by the PVDF and transduced into a voltage signal displayed by a 200 MHz oscilloscope (TDS 2024, Tektronix, Wilsonville, OR) triggered by a photodiode. The signals were amplified with a gain of 25 via a 350 MHz amplifier (SR445A, Stanford Research Systems, Sunnyvale, CA).

Cultured Melanoma Cells

Human malignant melanoma cell line HTB-67 SK-MEL-1(ATCC) was cultured in suspension with RPMI-1640 growth medium (Sigma), incubated at 37°C. The tissue sample was taken from the secondary metastatic site of rapidly spreading malignant melanoma facilitated by the lymphatic system. The patient was a 29 year old Caucasian male with primary skin carcinoma. Melanoma cells were isolated from culture by centrifugation and counted manually using a hemacytometer. Cells were washed in 0.9% saline. Cell samples underwent Fontana Masson melanin staining and cytospin analysis to determine a weakly melanotic culture with only 2% of cultured cells actively producing melanin.

Mononuclear Cell Separation

A sample of healthy, cancer free blood were drawn from a male subject. In addition, a blood sample was taken from a Stage IV melanoma patient who was presumably circulating melanoma cells. The samples were taken by venipuncture from the antecubital area of the arm in the amount of 30 ml using a standard blood draw procedure. Ethylenediaminetetraacetic acid (EDTA) liquid coated tubes were used for blood collection to inhibit clotting. Blood samples were stored in a refrigerated environment for no more than five hours before being processed.

A Ficoll-Hypaque separation technique was used to isolate the peripheral blood mononuclear cell (PBMC) layer from the whole blood samples. The Ficoll-Hypaque process employs a sugar compound of a specific density that separates specific blood components by a density gradient when centrifugal force is applied. Approximately 1 ml of Histopaque 1077 (Sigma-Aldrich Inc., St. Louis, MO) separation gradient was placed in Pyrex No. 9800 glass tubes (Corning Inc., Acton, MA). Approximately 7 ml of blood from the refrigerated samples were gently poured on top of the Histopaque 1077 and stopped with a rubber stopper. The sample tube was then placed in a 60 Hz 3400 rpm Vanguard 6500 centrifuge (Hamilton Bell Co., Montvale, NJ) and rotated for 10 minutes. The PBMC layer consisting of monocytes and lymphocytes (agranulocytes) separated out directly above the Histopaque layer and below the plasma.

Following separation, the differentiated PBMC layer was carefully removed using standard transfer pipets (Samco Scientific Corp., San Fernando, CA) and placed into 1.5 ml Flat Top Microcentrifuge Tubes (Fisher Scientific, Pittsburgh, PA). The PBMCs in the microcentrifuge tubes were washed in a saline solution and re-centrifuged for 5 minutes. This protocol was repeated until peripheral blood mononuclear cells were cleanly isolated.

These samples were tested in the photoacoustic flowmeter in the manner of the cultured melanoma cells.

III. Results

A typical representation of photoacoustic waves from saline and melanoma cells suspended in saline is shown in Figure **??**. While the saline sample showed a peak at about 1.5 μs, this was a constant background signal. The noise prior to 1 μs was interference from the laser system. The waveform in B shows photoacoustic waves generated in melanoma cells.

A. Photoacoustic Detection of Melanoma Cells

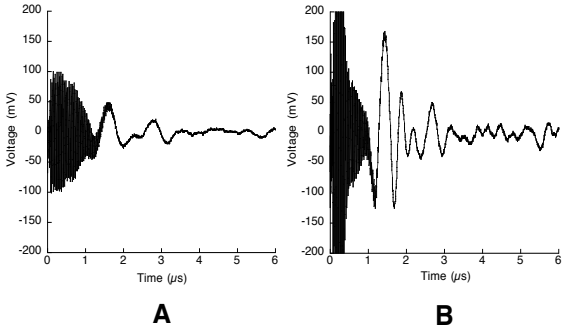

Fig. 2. **Melanoma suspended in saline.** A. Control waveform showing background signal from melanoma in saline. B. Photoacoustic waveform of isolate from saline spiked with 3.3×10^6 melanoma.

A set of serial dilutions of melanoma cells in saline showed the calibration curve in Figure 3. This calibration indicates a detection threshold of a single melanoma cell.

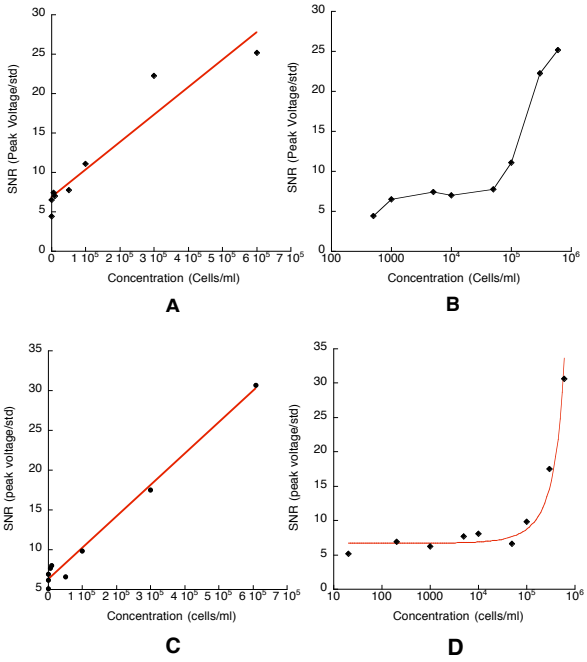

Fig. 3. **Serial dilutions of melanoma in saline.** A. Linear regression of peak voltages standardized over noise at 450 nm. B. Logarithmic representation of data shown in A. C. Linear regression of a second trial at 450 nm D. Exponential curve fit of data from C.

Figure 4 shows two photoacoustic waveforms. On the left, a flat line indicates the absence of melanoma cells in the sample from a healthy volunteer. The right waveform shows one of several transient photoacoustic signal from the Stage IV patient. While the waveform for the healthy volunteer was essentially flat, indicating no photoacoustic events and hence, no melanoma, the Stage IV patient's blood sample showed several transient photoacoustic spikes, corresponding to melanoma cells passing through the excitation laser beam. The duration of the photoacoustic spikes, approximately two seconds, corresponded to the laser repetition frequency, the system flow rate, and the number of averages that was set on the oscilloscope.

IV. Discussion

Further studies are required to definitively prove the relationship between melanoma and the PBMC isolate. The ability to detect single melanoma cells in the Stage IV patient may be more realistic than the capabilities of cultured melanoma cells. The cultured melanoma cell line used in these trials is a 30 year old line that, by melanin stain, shows a meager 2% of cells producing melanin, thus creating weak photoacoustic signals. It is certainly feasible that an active melanoma patient will produce much stronger melanin and hold a better ability to detect single cells. A larger study is

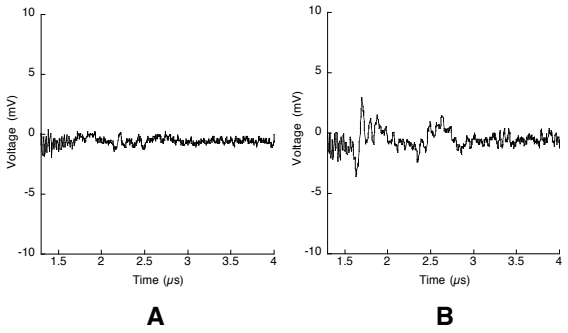

Fig. 4. **Melanoma detection in stage IV cancer patient.** A. The background photoacoustic signal from irradiating mononuclear cells in a healthy volunteer. B. The transient photoacoustic signal from a Stage IV melanoma patient. The duration of the signal corresponded to a particle flowing through the detection chamber at the given flow rate.

underway to gather important statistical data and to further prove the ability of photoacoustics to detect low levels of circulating tumor cells in the human blood stream.

The impact of this work is yet to be defined. First, this technology would give clinicians a powerful tool to monitor the disease state of advanced cancer patients and their response to therapy. Second, it will give basic scientists a means to study the process of metastasis and its relationship to CTC's. Finally, it will give cancer patients the ability to face their disease with immediate and accessible knowledge. The advancement of this technology will allow patients to definitively know if small concentrations of CTC's are present in their circulatory system long before such cells take root and form secondary tumors. It is the author's hope that this work will eventually be a viable clinical tool for the management of all cancer patients.

Acknowledgements

We acknowledge the support of the Department of Biological Engineering and the Christopher S. Bond Life Sciences Center at the University of Missouri for financial support and facilities during this study. We acknowledge the Wallace H. Coulter Foundation for providing assistance to Dr. Viator during the period of this research.

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